

SEQUENCE LISTING

<110> RIKEN

<120> Thermostable kanamycin nucleotidyltransferase and screening method of thermophiles
using thereof

<130> RJH11-072T

<160> 11

<170> PatentIn Ver. 2.0

<210> 1

<211> 253

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<213> Artificial Sequence

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<223> mutant enzyme obtained by introduction of point mutation into wild type
KNT gene and its expression

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His Glu Ile Lys Glu Arg Ile Leu Asp Lys Tyr Gly Asp Asp Val Lys

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Ala Ile Gly Val Tyr Gly Ser Leu Gly Arg Gln Thr Asp Gly Pro Tyr

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Ser Asp Ile Glu Met Met Cys Val Met Ser Thr Glu Glu Ala Glu Phe
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Ser His Glu Trp Thr Thr Gly Glu Trp Lys Val Glu Val Asn Phe Tyr
65 70 75 80

Ser Glu Glu Ile Leu Leu Asp Tyr Ala Ser Gln Val Glu Ser Asp Trp
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Pro Leu Thr His Gly Gln Phe Phe Ser Ile Leu Pro Ile Tyr Asp Ser
100 105 110

Gly Gly Tyr Leu Glu Lys Val Tyr Gln Thr Ala Lys Ser Val Glu Ala
115 120 125

Gln Lys Phe His Asp Ala Ile Cys Ala Leu Ile Val Glu Glu Leu Phe
130 135 140

Glu Tyr Ala Gly Lys Trp Arg Asn Ile Arg Val Gln Gly Pro Thr Thr
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Phe Leu Pro Ser Leu Thr Val Gln Val Ala Met Ala Gly Ala Met Leu
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Ile Gly Leu His His Arg Ile Cys Tyr Thr Thr Ser Ala Ser Val Leu
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Thr Glu Ala Val Lys Gln Ser Asp Leu Pro Ser Gly Tyr Asp His Leu
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Cys Gln Phe Val Met Ser Gly Gln Leu Ser Asp Ser Glu Lys Leu Leu
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His Gly Tyr Ile Val Asp Val Ser Lys Arg Ile Pro Phe
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into wild type KNT gene and its expression

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His Glu Ile Lys Glu Arg Ile Leu Asp Lys Tyr Gly Asp Asp Val Lys
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Ala Ile Gly Val Tyr Gly Ser Leu Gly Arg Gln Thr Asp Gly Pro Tyr
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Ser Asp Ile Glu Met Met Cys Val Met Ser Thr Glu Gly Ala Glu Phe
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Ser Tyr Glu Trp Thr Thr Gly Glu Trp Lys Ala Glu Val Asn Phe Tyr
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Ser Glu Glu Ile Leu Leu Asp Tyr Ala Ser Arg Val Glu Ser Asp Trp
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Pro Leu Thr His Gly Arg Phe Phe Ser Ile Leu Pro Ile Tyr Asp Pro
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Gly Gly Tyr Phe Glu Lys Val Tyr Gln Thr Ala Lys Ser Val Glu Ala
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Gln Lys Phe His Asp Ala Ile Cys Ala Leu Ile Val Glu Glu Leu Phe
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Glu Tyr Ala Gly Lys Trp Arg Asn Ile Arg Val Gln Gly Pro Thr Thr
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Phe Leu Pro Ser Leu Thr Val Gln Val Ala Met Ala Gly Ala Met Leu
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Ile Gly Leu His His Arg Ile Cys Tyr Thr Thr Ser Ala Ser Val Leu
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Thr Glu Ala Val Lys Gln Pro Asp Leu Pro Ser Gly Tyr Asp His Leu
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Cys Gln Leu Val Met Ser Gly Gln Leu Ser Asp Ser Glu Lys Leu Leu
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Glu Ser Leu Glu Asn Phe Trp Asn Gly Ile Gln Glu Trp Thr Glu Arg

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His Gly Tyr Ile Val Asp Val Ser Lys Arg Ile Pro Phe

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KNT gene and its expression

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His Glu Ile Lys Glu Arg Ile Leu Asp Lys Tyr Gly Asp Asp Val Lys

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Ala Ile Gly Val Tyr Gly Ser Leu Gly Arg Gln Thr Asp Gly Pro Tyr

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Ser Asp Ile Glu Met Met Cys Val Leu Ser Thr Glu Gly Val Glu Phe

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Ser Tyr Glu Trp Thr Thr Gly Glu Trp Lys Ala Glu Val Asn Phe Tyr

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Ser Glu Glu Ile Leu Leu Asp Tyr Ala Ser Arg Val Glu Pro Asp Trp

85

90

95

Pro Leu Thr His Gly Arg Phe Phe Ser Ile Leu Pro Ile Tyr Asp Pro

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Gly Gly Tyr Phe Glu Lys Val Tyr Gln Thr Ala Lys Ser Val Glu Ala

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Gln Lys Phe His Asp Ala Ile Cys Ala Leu Ile Val Glu Glu Leu Phe

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Glu Tyr Ala Gly Lys Trp Arg Asn Ile Arg Val Gln Gly Pro Thr Thr

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Phe Leu Pro Ser Leu Thr Val Gln Val Ala Met Ala Gly Ala Met Leu

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175

Ile Gly Leu His His Arg Ile Cys Tyr Thr Thr Ser Ala Ser Val Leu

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Thr Glu Ala Val Lys Gln Pro Asp Leu Pro Pro Gly Tyr Val Gln Leu

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Cys Gln Leu Val Met Ser Gly Gln Leu Ser Asp Pro Glu Lys Leu Leu

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Glu Ser Leu Glu Asn Phe Trp Asn Gly Val Gln Glu Trp Ala Glu Arg

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His Gly Tyr Ile Val Asp Val Ser Lys Arg Ile Pro Phe

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<223> Description of Artificial Sequence: 3'-Primer for PCR amplification

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<223> Description of Artificial Sequence: 5'-Primer for PCR amplification

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<223> Description of Artificial Sequence:5'-Primer for subcloning of KT3-11 and HTK

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His Glu Ile Lys Glu Arg Ile Leu Asp Lys Tyr Gly Asp Asp Val Lys

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Ala Ile Gly Val Tyr Gly Ser Leu Gly Arg Gln Thr Asp Gly Pro Tyr

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tcg gat att gag atg atg tgt gtc atg tca aca gag gaa gca gag ttc 192

Ser Asp Ile Glu Met Met Cys Val Met Ser Thr Glu Glu Ala Glu Phe

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agc cat gaa tgg aca acc ggt gag tgg aag gtg gaa gtg aat ttt gat 240
 Ser His Glu Trp Thr Thr Gly Glu Trp Lys Val Glu Val Asn Phe Asp
 65 70 75 80

agc gaa gag att cta cta gat tat gca tct cag gtg gaa tca gat tgg 288
 Ser Glu Glu Ile Leu Leu Asp Tyr Ala Ser Gln Val Glu Ser Asp Trp
 85 90 95

ccg ctt aca cat ggt caa ttt ttc tct att ttg ccg att tat gat tca 336
 Pro Leu Thr His Gly Gln Phe Phe Ser Ile Leu Pro Ile Tyr Asp Ser
 100 105 110

ggt gga tac tta gag aaa gtg tat caa act gct aaa tcg gta gaa gcc 384
 Gly Gly Tyr Leu Glu Lys Val Tyr Gln Thr Ala Lys Ser Val Glu Ala
 115 120 125

caa acg ttc cac gat gcg att tgt gcc ctt atc gta gaa gag ctg ttt 432
 Gln Thr Phe His Asp Ala Ile Cys Ala Leu Ile Val Glu Glu Leu Phe
 130 135 140

gaa tat gca ggc aaa tgg cgt aat att cgt gtg caa gga ccg aca aca 480
 Glu Tyr Ala Gly Lys Trp Arg Asn Ile Arg Val Gln Gly Pro Thr Thr
 145 150 155 160

ttt cta cca tcc ttg act gta cag gta gca atg gca ggt gcc atg ttg 528
 Phe Leu Pro Ser Leu Thr Val Gln Val Ala Met Ala Gly Ala Met Leu
 165 170 175

att ggt ctg cat cat cgc atc tgt tat acg acg agc gct tcg gtc tta 576
 Ile Gly Leu His His Arg Ile Cys Tyr Thr Thr Ser Ala Ser Val Leu

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Thr Glu Ala Val Lys Gln Ser Asp Leu Pro Ser Gly Tyr Asp His Leu

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tgc cag ttc gta atg tct ggt caa ctt tcc gac tct gag aaa ctt ctg 672

Cys Gln Phe Val Met Ser Gly Gln Leu Ser Asp Ser Glu Lys Leu Leu

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Glu Ser Leu Glu Asn Phe Trp Asn Gly Ile Gln Glu Trp Thr Glu Arg

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His Glu Ile Lys Glu Arg Ile Leu Asp Lys Tyr Gly Asp Asp Val Lys

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Ala Ile Gly Val Tyr Gly Ser Leu Gly Arg Gln Thr Asp Gly Pro Tyr

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Ser Asp Ile Glu Met Met Cys Val Met Ser Thr Glu Glu Ala Glu Phe

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Ser His Glu Trp Thr Thr Gly Glu Trp Lys Val Glu Val Asn Phe Asp

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Ser Glu Glu Ile Leu Leu Asp Tyr Ala Ser Gln Val Glu Ser Asp Trp

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Pro Leu Thr His Gly Gln Phe Phe Ser Ile Leu Pro Ile Tyr Asp Ser

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Gly Gly Tyr Leu Glu Lys Val Tyr Gln Thr Ala Lys Ser Val Glu Ala

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Gln Thr Phe His Asp Ala Ile Cys Ala Leu Ile Val Glu Glu Leu Phe

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Glu Tyr Ala Gly Lys Trp Arg Asn Ile Arg Val Gln Gly Pro Thr Thr

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Phe Leu Pro Ser Leu Thr Val Gln Val Ala Met Ala Gly Ala Met Leu

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170

175

Ile Gly Leu His His Arg Ile Cys Tyr Thr Thr Ser Ala Ser Val Leu

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Thr Glu Ala Val Lys Gln Ser Asp Leu Pro Ser Gly Tyr Asp His Leu

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205

Cys Gln Phe Val Met Ser Gly Gln Leu Ser Asp Ser Glu Lys Leu Leu
210 215 220

Glu Ser Leu Glu Asn Phe Trp Asn Gly Ile Gln Glu Trp Thr Glu Arg
225 230 235 240

His Gly Tyr Ile Val Asp Val Ser Lys Arg Ile Pro Phe
245 250

[Sequence Listing Free Text]

SE ID NO:1: mutant enzyme obtained by introduction of point mutation into wild type K
NT gene of Staphylococcus aureus and its expression

SE ID NO:2: mutant enzyme obtained by introduction of point mutation
into wild type KNT gene of Staphylococcus aureus and its expression

SE ID NO:3: mutant enzyme obtained by introduction of point mutation into wild type K
NT gene of Staphylococcus aureus and its expression

SE ID NO:4: 5'-Primer for PCR amplification

SE ID NO:5: 3'-Primer for PCR amplification

SE ID NO:6: 5'-Primer for PCR amplification

SE ID NO:7: 5'-Primer for subcloning of WT*

SE ID NO:8: 5'-Primer for subcloning of KT3-11 and HTK

SE ID NO:9: 3'-Primer for subcloning

[Brief Description of the Drawings]

[Figure 1]

Figure 1 indicates the restriction maps for each of plasmids (a)pYK134, (b)pTT8, and (c)pJHK1.

[Figure 2]

Figure 2 indicates the thermostability of KNT.

- (A) The heat denaturation of WT* (\square), KT3-11 (Δ) and HTK (\circ) was monitored on a CD at 222nm and recorded. Measurement conditions were: protein concentration 0.8 μ M; 50mM potassium phosphate buffer containing 0.1M KCl; and pH of 7.0.

$$\text{Degree of Denaturation (\%)} = (\theta_{222}^T - \theta_{222}^N) / (\theta_{222}^D - \theta_{222}^N)$$

(θ_{222}^T is the average residue molecular ellipticity at T°C, at 222nm and θ_{222}^N and θ_{222}^D are the average residue molecular ellipticities at 222nm for the non-denatured and denatured enzyme, respectively.)

- (B) Heat Inactivation: Enzyme solution is heated for ten minutes at the designated temperature. After cooling, activity is measured at 25°C. Heat treatment was conducted with a protein concentration of 1.2 μ M and using the same buffer as in the CD measurement. The values for each enzyme are expressed as a ratio in comparison to the respective non-heat-treated enzyme. Each numerical value has a standard deviation of $\pm 10\%$

[Figure 3]

Figure 3 indicates a 3-dimensional representation of the structure of KNT having the Asp80Tyr mutation (KT3-11 and HTK). KNT is a homodimer, and the positions and the residue numbers of modified residues are indicated for only one of the subunits. The mutated residues of KT3-11, the additional 9 mutated residues of HTK, kanamycin, and adenosine 5'- α , β -methylene triphosphate which is an analog of ATP, are indicated. This figure was prepared using MOLSCRIPT (Per Kraulis, Department of Molecular Biology, Uppsala University, Sweden.)

[Figure 4]

Figure 4 indicates a restriction map for plasmid pJHK3.

[Designation of Document] Abstract

[Problem to be solved]

To obtain a selective marker suitable for screening of thermophilic bacteria such as *Thermus thermophilus*. *T. thermophilus* are good research materials for investigating the interrelation between enzyme structures and functions since they are stable at extreme pH, crystallize easily and are easy-to-handle.

[Means for Solving the Problem]

A novel kanamycin nucleotidyltransferase with markedly improved thermostability, a selective marker using the same, and a screening method for thermophilic bacteria such as *Thermus thermophilus* using said selective marker, are provided.

[Representative Drawing] Figure 2.